Ex ovo Chicken Culturing: The Effect Ethanol and Vessel Shape have on the Development of the Chick Embryo

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Introduction

Background:
- Scientists have been growing chicks outside of eggshells (ex-ovo) for decades
- Various ex-ovo vessels were published in the past:
  - Plastic bag (Elliot et al. 1971)
  - Weigh boat (Dorrel et al. 2014) **(Figure 2.)**
  - Cube with PDMS, material of contact lenses (Huang et al. 2014) **(Figure 3.)**
  - Cup with plastic a film across it, suspending yolk (Tahara et al. 2014) **(Figure 4.)**

Long-term goal:
- Create novel culturing vessel through combining benefits from previously published vessels to optimize *ex ovo* culture condition for chick embryo development.
- Use this model to serve as a platform for testing the ability of tissue engineering scaffold to support blood vessel innervation for sustaining cell growth after implantation

Previous accomplishment:
- Designed novel truncated hexagonal pyramid (THP) vessel **(Figure 5.)**
  - PDMS sidings that are transparent and oxygen permeable
  - Easy to view and manipulate embryo
  - Static culture structure with bigger opening and tapered ends

Observation:
- Some chicks were seen to have ventral wall deformation. **(Figure 12A.)**

Project Goal:
Determine the cause of the observed ventral wall deformity

Literature survey:
- Ethanol has been reported to deter the migration of trunk cells in developing embryos resulting in failure of midline closure (Zagory et al, 2004)
- Various human ventral wall deformities have been reported including: gastroschisis, omphalocele, ectopia cordis

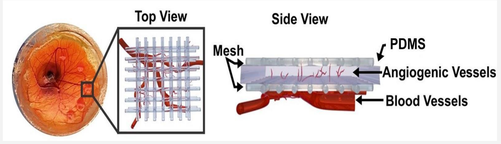

Figure 1. CAM layer used to support cells on a scaffold.

Figure 2. Dorrel et al. weigh boat vessel.

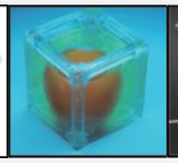

Figure 3. Huang et al. cubic PDMS vessel.

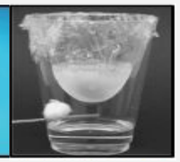

Figure 4. Tahara et al. PMP hammock vessel.

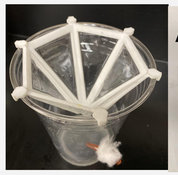

Figure 5. Truncated hexagonal pyramid (THP) culturing vessel.

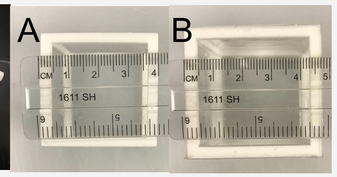

Figure 6. Cube diameter measurements. Image A depicts the opening of Huang et al's cube vessel (4.0 cm). Image B depicts the opening of our cube vessel (4.8 cm).

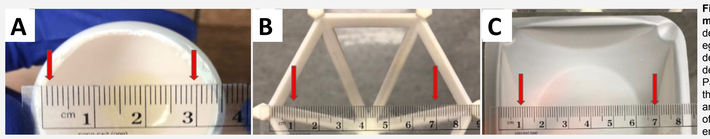

Figure 7. Vessel diameter measurements. Panel A depicts opening of normal eggshell (3.5 cm). Panel B depicts the opening of designed THP vessel (8.5 cm). Panel C depicts the opening of the weigh boat (8 cm). The red arrows indicate the either side of the top brim of where the embryo usually fills up to.

Hypothesis and Aims

Hypothesis:

1. The 70% ethanol used for vessel sterilization, is causing the observed ventral wall deformity in the chick embryos **(Figure 12A.)**

2. The size of the ex ovo culturing vessels is causing the observed ventral wall deformity in the chick embryos **(Figures 6 and 7).**

Aims:

- Culture chick embryos in the THP and weigh boat *ex ovo* vessels and treat with 250ul of water (control) vs 250ul of 30% ethanol (experiment) after 55-56hr incubation.
- Collect and compare ventral wall closure as well as developmental markers between untreated and treated ethanol chicks.
- Culture chick embryos in THP and cube *ex ovo* vessels
- Collect and compare ventral wall closure between THP and cube vessel chicks.

Materials and Methods

Cracking & Transferring the embryo:

1. Remove egg from incubator at 55-56 hours and sterilize shell with 70% EtOH.
2. Twist a scalpel to carve a small hole at the top of the eggshell (wide pole facing up) **(Figure 9A.)**
3. Tilt the egg to the side to ensure yolk and blastoderm are located away from the carved hole
4. Using a 5cc syringe, insert through hole and draw out 10mL albumen making sure to avoid yolk location **(Figure 9B.)**
5. Place 10mL albumen into weigh boat (this will provide a cushion for the yolk to land on top of)
6. Using a rotary tool, gently carve away the wide pole of the egg (rotary tool to be used near the top quadrant) **(Figure 9C.)**
7. With needle-point tweezers, remove the top of the eggshell and visualize the viability of the embryo
8. Gently tilt eggshell in correlation with weigh boat so that yolk and remaining albumen transfer into the weigh boat **(Figure 9D.)**
9. Transfer back and forth between weigh boats until the blastoderm is successfully on top.

Ethanol Induction

1. Draw small aliquot (250uL) of prepared EtOH solution at 30% concentration with P200 pipette
2. Release concentrated solution into albumin.
3. Label and place vessel into incubator at standard conditions
   37 degrees Celsius
   60-70% humidity

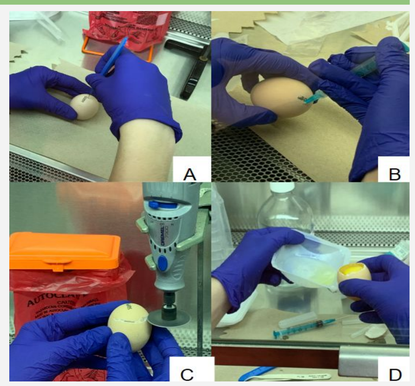

Figure 9. Transfer of Embryo to *ex ovo* Weigh Boat Culture Vessel. A) Tilting the egg to avoid contact and consequential rupturing of vitelline membrane surrounding yolk. B) Use of rotary tool near the top quadrant of egg avoiding albumen splatter or contact with yolk
C) Use of rotary tool to obtain access to embryo
D) Act of pouring the embryo into the weigh boat for a successful transfer

Results

Figure 10. Progression of chick embryo development in weigh boat *ex ovo* culture vessel with ethanol treatment. Ethanol induced weigh boat model derived from [Dorrell *et al*] allows the chick embryo to develop in an open system for easy viewing and manipulation. Model above represents a chick that has been exposed to 150uL of 30% EtOH solution at 55-56hr post incubation. E4 represents embryonic day 4, E5 represents embryonic day 5 and so on so forth.

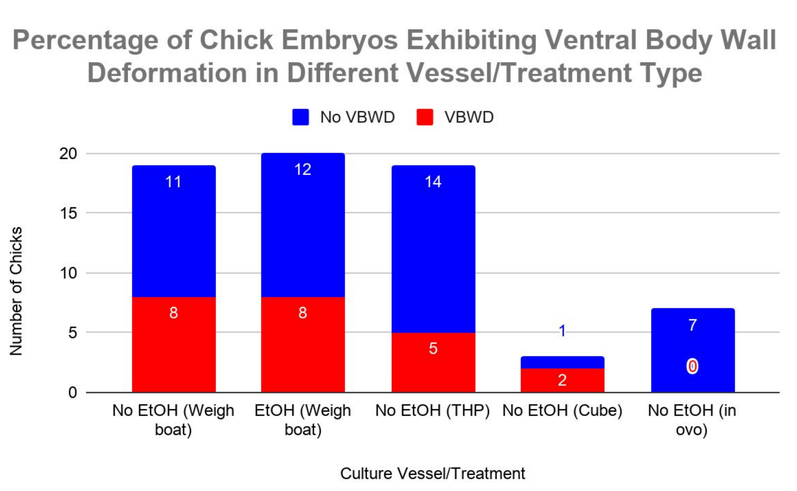

Figure 11. Percentage of chicks which exhibited a ventral body wall deformation. The graph shows the percentages of the chicks which had an apparent VBWD when cultured in the weigh boat (with/without EtOH), THP (No EtOH), cube vessels (No EtOH), and in ovo (No EtOH). Data from the Spring '19 and Fall '19 (n=68 chicks noted upon) shows that ventral wall closure failed in 42.1% of the 19 chicks in the weigh boat with no EtOH, 40.0% of the 20 chicks in the weigh boat with EtOH added, 26.3% of the 19 chicks in the THP vessel with no EtOH, 66.7% of the three chicks in the cube vessel with no EtOH, and 0% of the 7 chicks *in ovo* exhibited this deformity.

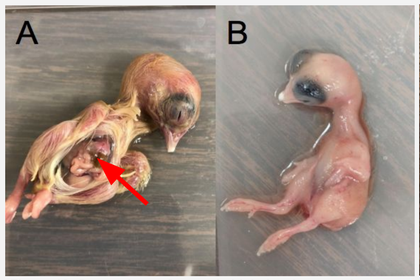

Figure 12. Comparison of normal chick development *in ovo* vs EtOH treated chick development *ex ovo*. Panel A is an embryonic day 18 chick having been exposed to EtOH solution *ex ovo*. A clear ventral wall deformity is present. Panel B is an embryonic day 13 chick developed *in ovo*. No clear ventral wall deformity exists.

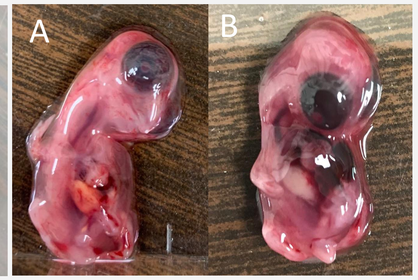

Figure 13. Ventral body wall deformity present in chick embryos without EtOH treatment developed in cube and THP *ex ovo* culture vessels. A) Chick depicted was cultured in a cubic vessel and was imaged on embryonic day 9. It has a complete VBWD present. B) Chick depicted was cultured in a THP vessel and was imaged on embryonic day 8. It has a complete VBWD present.

Figure 14. Ventral body wall deformity present in chick embryos without EtOH treatment developed in weigh boat *ex ovo* culture vessel. Panel A is an embryonic day 14 chick not induced with EtOH grown *ex ovo.* Panel B and C are embryonic day 16 chicks not induced with EtOH grown *ex ovo.* A clear ventral wall deformity is present in all of the chicks pictured above.

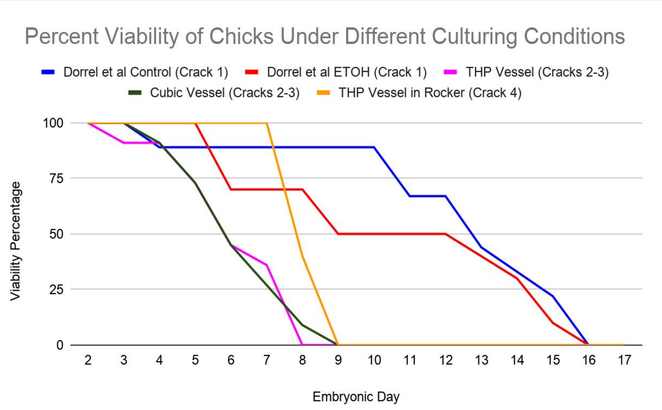

Figure 15. The comparison of viability when chick embryos are cultured in different vessels. This graph shows the viability percentages of the chick embryos when grown in weigh boat, THP and cube vessels. The red and blue lines represent weigh boat chicks, blue with no EtOH treatment and red with EtOH treatment. The yellow line represents THP chicks. The green line represents cube chicks. The orange line represents the THP chicks that were placed on the rocker. All of the lines represent chicks cracked on embryonic day 2, except for the orange line which were cracked on embryonic day 3. Based on the data collected, the weigh boat chicks had the highest viability rate and were seen to survive the longest.

Discussion & Conclusion

Discussion

~Prior Research~
- On cultivating chick embryos using an *ex ovo* model, we noticed failure in ventral body wall closure.
- It was hypothesized that ethanol sterilization technique causes the phenotypic ventral body wall deformity (VBWD) observed.
- Literature was reviewed to better understand ventral wall defect terminology and etiology in human.
- Previously referred to this deformation as an omphalocele, then gastroschisis, but decided to use the general term VBWD.
- Both upper VBWD, including the heart developing outside the chest cavity, and lower VBWD, including intestines developing outside the thoracic cavity were observed, sometimes with both deformations present.
- It is believed that the malformation occurring in the chicks involves the process of lateral mesodermal wall closure along the midline.

~Fall 2019~
- Tested the ethanol sterilization and VBWD hypothesis.
- Tested the effects of different shaped vessels on VBWD.
- After supplementing chicks with 250uL of 30% EtOH and in 250uL of distilled H2O, the rates of VBWD were consistent leading to the conclusion that EtOH was not responsible for the failure of the lateral mesodermal wall closure.
- It was observed that the vessels are shaped significantly different than the natural shape of an egg so it was hypothesized that vessel shape may be causing VBWD.
- Chicks were incubated in weigh boats, THP vessels, and cube vessels. All three vessel types exhibited VBWD.
- At this point in time no conclusions have been made regarding vessel shape but the data is showing VBWD regardless of vessel shape.

Conclusion

Two Hypotheses:
1. Ethanol Exposure causes VBWD
2. Vessel Shape causes VBWD

- The data failed to prove that ethanol is the cause of the ventral wall deformity based on the results that the control chicks are also showing the deformity **(Figure 11).**
- Ventral body wall development between *ex ovo* chick model and *in ovo* control group were compared **(Figure 12).**
- Ventral body wall deformation was discredited as a normal process in development as the ventral wall is completely sealed early on in development of the chick embryo **(Figure 17).**
- It was possible that VBWD could be an artifact from dissection when we are manipulating the chick embryo to take developmental markers. We created a standardized method for dissecting embryos and determined this is not a cause of the VBWD.
- The data failed to prove that vessel shape and excess volumetric/circumferential space of the weigh boat, cube, or THP could be causing VBWD (**Figure 17**).
- The positioning of the embryo could be important when developing and the extra space in the vessels could cause strain and prevent the ventral body wall from fusing.
- The cube vessel was still showing VBWD **(Figure 13).**
- We noticed a viability difference between the three vessels tested. Chicks developed in the Dorrell et al weigh boat exhibit the highest viability (**Figure 15**).
- In the future it will be important to maintain the Buff Orpington strain in order to keep our results consistent.

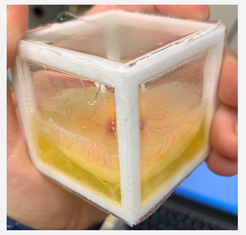

Figure 15. Development of chick embryo in cube vessel. Blood vessels have development down the side of PDM showing that it is oxygen permeable.

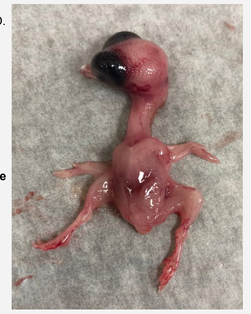

Figure 17. Buff orpington chick embryo developed *in ovo*. Pictured is an embryonic day 11 chick that was grown *in ovo* and was not exposed to ethanol.

Future Research

- Replicate the Huang et al cube to test whether vessel shape is causing the VBWD due to the excess volumetric space in the larger cube, weigh boat, and THP vessels (Figure 6 and 7).
- Supply embryos with oxygen to see if maintaining a constant 21% oxygen level within the incubator has an effect on development.
- Continue testing photobiomodulation on the CAM layer to stimulate angiogenesis.
- Test scaffolds on the CAM layer.
- Incubate eggs for 72 hours instead of 55 hours to further development before cracking into *ex ovo* culture vessel.
- Seal vessels to test whether negative pressure affects development.
- Turning *ex ovo* vessels to test its effects on VBWD and development.

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